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<b>(54) Title:</b> METHOD FOR SIMULTANEOUS IDENTIFICATION OF DIFFERENTIALLY EXPRESSED mRNAs AND MEASUREMENT OF RELATIVE CONCENTRATIONS		
<b>(57) Abstract</b>  An improved method for the simultaneous sequence-specific identification of mRNAs in an mRNA population allows the visualization of nearly every mRNA expressed by a tissue as a distinct band on a gel whose intensity corresponds roughly to the concentration of the mRNA. In general, the method comprises the formation of cDNA using anchor primers to fix a 3'-endpoint, producing cloned inserts from the cDNA in a vector containing a bacteriophage-specific promoter for subsequent RNA synthesis, generating linearized fragments of the cloned inserts, preparing cRNA, transcribing cDNA from the cRNA using a set of primers, and performing PCR using a 3'-primer whose sequence is derived from the vector and a set of 5'-primers that is derived from the primers used for transcription of cDNA from cRNA. The method can identify changes in expression of mRNA associated with the administration of drugs or with physiological or pathological conditions.		

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METHOD FOR SIMULTANEOUS IDENTIFICATION  
OF DIFFERENTIALLY EXPRESSED mRNAs AND MEASUREMENT  
OF RELATIVE CONCENTRATIONS

BACKGROUND OF THE INVENTION

This invention is directed to methods for simultaneous identification of differentially expressed mRNAs, as well as measurements of their relative concentrations.

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An ultimate goal of biochemical research ought to be a complete characterization of the protein molecules that make up an organism. This would include their identification, sequence determination,  
10 demonstration of their anatomical sites of expression, elucidation of their biochemical activities, and understanding of how these activities determine organismic physiology. For medical applications, the description should also include information about how the  
15 concentration of each protein changes in response to pharmaceutical or toxic agents.

Let us consider the scope of the problem: How many genes are there? The issue of how many genes are  
20 expressed in a mammal is still unsettled after at least two decades of study. There are few direct studies that address patterns of gene expression in different tissues. Mutational load studies (J.O. Bishop, "The Gene Numbers Game," Cell 2:81-86 (1974); T. Ohta & M. Kimura,  
25 "Functional Organization of Genetic Material as a Product of Molecular Evolution," Nature 223:118-119 (1971)) have suggested that there are between  $3 \times 10^4$  and  $10^5$  essential genes.

30

Before cDNA cloning techniques, information on

mixed populations of RNA molecules with different

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specificities in abundances. To an unexpected extent, early analog complexity studies were distorted by hidden complications of the fact that the molecules in each tissue that make up most of its mRNA mass comprise only a small fraction of its total complexity. Later, cDNA cloning allowed digital measurements (i.e., sequence-specific measurements on individual species) to be made; hence, more recent concepts about mRNA expression are based upon actual observations of individual RNA species.

10

Brain, liver, and kidney are the mammalian tissues that have been most extensively studied by analog RNA complexity measurements. The lowest estimates of complexity are those of Hastie and Bishop (N.D. Hastie & J. B. Bishop, "The Expression of Three Abundance Classes of Messenger RNA in Mouse Tissues," Cell 9:761-774 (1976)), who suggested that  $26 \times 10^6$  nucleotides of the  $3 \times 10^9$  base pair rodent genome were expressed in brain,  $23 \times 10^6$  in liver, and  $22 \times 10^6$  in kidney, with nearly complete overlap in RNA sets. This indicates a very minimal number of tissue-specific mRNAs. However, experience has shown that these values must clearly be underestimates, because many mRNA molecules, which were probably of abundances below the detection limits of this early study, have been shown to be expressed in brain but detectable in neither liver nor kidney. Many other researchers (J.A. Bantle & W.E. Hahn, "Complexity and Characterization of Polyadenylated RNA in the Mouse Brain," Cell 8:139-150 (1976); D.M. Chikaraishi, "Complexity of Cytoplasmic Polyadenylated and Non-Adenylated Rat Brain Ribonucleic Acids," Biochemistry 18:3249-3256 (1979)) have measured analog complexities of between  $100\text{--}200 \times 10^6$  nucleotides in brain, and 2-to-3-fold

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values have been supported by digital cloning studies

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(R.J. Milner & J.G. Sutcliffe, "Gene Expression in Rat Brain," Nucl. Acids Res. 11:5497-5520 (1983)).

5        Analog measurements on bulk mRNA suggested that  
the average mRNA length was between 1400-1900  
nucleotides. In a systematic digital analysis of brain  
mRNA length using 200 randomly selected brain cDNAs to  
measure RNA size by northern blotting (Milner &  
Sutcliffe, supra), it was found that, when the mRNA size  
10      data were weighted for RNA prevalence, the average length  
was 1790 nucleotides, the same as that determined by  
analog measurements. However, the mRNAs that made up  
most of the brain mRNA complexity had an average length  
of 5000 nucleotides. Not only were the rarer brain RNAs  
15      longer, but they tended to be brain specific, while the  
more prevalent brain mRNAs were more ubiquitously  
expressed and were much shorter on average.

20        These concepts about mRNA lengths have been  
corroborated more recently from the length of brain mRNA  
whose sequences have been determined (J.G. Sutcliffe,  
"mRNA in the Mammalian Central Nervous System," Annu.  
Rev. Neurosci. 11:157-198 (1988)). Thus, the  $1-2 \times 10^8$   
nucleotide complexity and 5000-nucleotide average mRNA  
25      length calculates to an estimated 30,000 mRNAs expressed  
in the brain, of which about 2/3 are not detected in  
liver or kidney. Brain apparently accounts for a  
considerable portion of the tissue-specific genes of  
mammals. Most brain mRNAs are expressed at low  
30      concentration. There are no total-mammal mRNA complexity  
measurements, nor is it yet known whether 5000  
nucleotides is a good mRNA-length estimate for non-neural  
tissues. A reasonable estimate of total gene number

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What is most needed to advance by a chemical understanding of physiological function is a menu of protein sequences encoded by the genome plus the cell types in which each is expressed. At present, protein sequences can be reliably deduced only from cDNAs, not from genes, because of the presence of the intervening sequences (introns) in the genomic sequences. Even the complete nucleotide sequence of a mammalian genome will not substitute for characterization of its expressed sequences. Therefore, a systematic strategy for collecting transcribed sequences and demonstrating their sites of expression is needed. Such a strategy would be of particular use in determining sequences expressed differentially within the brain. It is necessarily an eventual goal of such a study to achieve closure; that is, to identify all mRNAs. Closure can be difficult to obtain due to the differing prevalence of various mRNAs and the large number of distinct mRNAs expressed by many distinct tissues. The effort to obtain it allows one to obtain a progressively more reliable description of the dimensions of gene space.

Studies carried out in the laboratory of Craig Venter (M.D. Adams et al., "Complementary DNA Sequencing: Expressed Sequence Tags and Human Genome Project," Science 252:1651-1656 (1991); M.D. Adams et al., "Sequence Identification of 2,375 Human Brain Genes," Nature 355:632-634 (1992)) have resulted in the isolation of randomly chosen cDNA clones of human brain mRNAs, the determination of short single-pass sequences of their 3'-ends, about 300 base pairs, and a compilation of some 2500 of these as a database of "expressed sequence tags." This database, while useful, fails to provide any

overall pattern of expression within regions of brain and

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other tissues and in response to various paradigms, such as various physiological or pathological states or the effects of drug treatment, rather than simply their expression in a single tissue.

5

Other work has focused on the use of the polymerase chain reaction (PCR) to establish a database. Williams et al. (J.G.K. Williams et al., "DNA Polymorphisms Amplified by Arbitrary Primers Are Useful as Genetic Markers," Nucl. Acids Res. 18:6531-6535 (1990)) and Welsh & McClelland (J. Welsh & McClelland, "Genomic Fingerprinting Using Arbitrarily Primed PCR and a Matrix of Pairwise Combinations of Primers," Nucl. Acids Res. 18:7213-7218 (1990)) showed that single 10-mer primers of arbitrarily chosen sequences, i.e., any 10-mer primer off the shelf, when used for PCR with complex DNA templates such as human, plant, yeast, or bacterial genomic DNA, gave rise to an array of PCR products. The priming events were demonstrated to involve incomplete complementarity between the primer and the template DNA. Presumably, partially mismatched primer-binding sites are randomly distributed through the genome. Occasionally, two of these sites in opposing orientation were located closely enough together to give rise to a PCR product band. There were on average 8-10 products, which varied in size from about 0.4 to about 4 kb and had different mobilities for each primer. The array of PCR products exhibited differences among individuals of the same species. These authors proposed that the single arbitrary primers could be used to produce restriction fragment length polymorphism (RFLP)-like information for genetic studies. Others have applied this technology (S.R. Woodward et al., "Random Sequence Oligonucleotide

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J.H. Nadeau et al., "Multilocus Markers for Mouse Genome

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Analysis: PCR Amplification Based on Single Primers of Arbitrary Nucleotide Sequence," Mamm. Genome 3:55-64 (1992)).

5                   Two groups (J. Welsh et al., "Arbitrarily Primed PCR Fingerprinting of RNA," Nucl. Acids Res. 20:4965-4970 (1992); P. Liang & A.B. Pardee, "Differential Display of Eukaryotic Messenger RNA by Means of the Polymerase Chain Reaction," Science 257:967-  
10 971 (1992)) adapted the method to compare mRNA populations. In the study of Liang and Pardee, this method, called mRNA differential display, was used to compare the population of mRNAs expressed by two related cell types, normal and tumorigenic mouse A31 cells. For  
15 each experiment, they used one arbitrary 10-mer as the 5'-primer and an oligonucleotide complementary to a subset of poly A tails as a 3' anchor primer, performing PCR amplification in the presence of <sup>35</sup>S-dNTPs on cDNAs prepared from the two cell types. The products were  
20 resolved on sequencing gels and 50-100 bands ranging from 100-500 nucleotides were observed. The bands presumably resulted from amplification of cDNAs corresponding to the 3'-ends of mRNAs that contain the complement of the 3' anchor primer and a partially mismatched 5' primer site,  
25 as had been observed on genomic DNA templates. For each primer pair, the pattern of bands amplified from the two cDNAs was similar, with the intensities of about 80% of the bands being indistinguishable. Some of the bands were more intense in one or the other of the PCR samples;  
30 a few were detected in only one of the two samples.

Further studies (P. Liang et al., "Distribution and Cloning of Eukaryotic mRNAs by Means of Differential

works with low concentrations of input RNA although it



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is not quantitative for rarer species), and the specificity resides primarily in the last nucleotide of the 3' anchor primer. At least a third of identified differentially detected PCR products correspond to  
5 differentially expressed RNAs, with a false positive rate of at least 25%.

If all of the 50,000 to 100,000 mRNAs of the mammal were accessible to this arbitrary-primer PCR  
10 approach, then about 80-95 5' arbitrary primers and 12 3' anchor primers would be required in about 1000 PCR panels and gels to give a likelihood, calculated by the Poisson distribution, that about two-thirds of these mRNAs would be identified.

15 It is unlikely that all mRNAs are amenable to detection by this method for the following reasons. For an mRNA to surface in such a survey, it must be prevalent enough to produce a signal on the autoradiograph and  
20 contain a sequence in its 3' 500 nucleotides capable of serving as a site for mismatched primer binding and priming. The more prevalent an individual mRNA species, the more likely it would be to generate a product. Thus, prevalent species may give bands with many different  
25 arbitrary primers. Because this latter property would contain an unpredictable element of chance based on selection of the arbitrary primers, it would be difficult to approach closure by the arbitrary primer method. Also, for the information to be portable from one  
30 laboratory to another and reliable, the mismatched priming must be highly reproducible under different laboratory conditions using different PCR machines, with the resulting slight variation in reaction conditions. As

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obtained by the Liang & Pardee differential display method.

- There is therefore a need for an improved method of differential display of mRNA species that reduces the uncertain aspect of 5'-end generation and allows data to be absolutely reproducible in different settings. Preferably, such a method does not depend on potentially irreproducible mismatched priming.
- 5      method of differential display of mRNA species that reduces the uncertain aspect of 5'-end generation and allows data to be absolutely reproducible in different settings. Preferably, such a method does not depend on potentially irreproducible mismatched priming.
- 10     Preferably, such a method reduces the number of PCR panels and gels required for a complete survey and allows double-strand sequence data to be rapidly accumulated. Preferably, such an improved method also reduces, if not eliminates, the number of concurrent signals obtained
- 15     from the same species of mRNA.

#### SUMMARY

We have developed an improved method for the simultaneous sequence-specific identification of mRNAs in a mRNA population. In general, this method comprises:

20     a mRNA population. In general, this method comprises:

- (1) preparing double-stranded cDNAs from a mRNA population using a mixture of 12 anchor primers, the anchor primers each including: (i) a tract of from 7 to 40 T residues; (ii) a site for cleavage by a restriction endonuclease that recognizes more than six bases, the site for cleavage being located to the 5'-side of the tract of T residues; (iii) a stuffer segment of from 4 to 40 nucleotides, the stuffer segment being located to the 5'-side of the site for cleavage by the restriction endonuclease; and (iv) phasing residues -V-N located at the 3' end of each of the anchor primers, wherein V is a deoxyribonucleotide selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from
- 25     endonuclease that recognizes more than six bases, the site for cleavage being located to the 5'-side of the tract of T residues; (iii) a stuffer segment of from 4 to 40 nucleotides, the stuffer segment being located to the 5'-side of the site for cleavage by the restriction
- 30     endonuclease; and (iv) phasing residues -V-N located at the 3' end of each of the anchor primers, wherein V is a deoxyribonucleotide selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from

V and N;

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(2) producing cloned inserts from a suitable host cell that has been transformed by a vector, the vector having the cDNA sample that has been cleaved with a first restriction endonuclease and a second restriction endonuclease inserted therein, the cleaved cDNA sample being inserted in the vector in an orientation that is antisense with respect to a bacteriophage-specific promoter within the vector, the first restriction endonuclease recognizing a four-nucleotide sequence and the second restriction endonuclease cleaving at a single site within each member of the mixture of anchor primers;

(3) generating linearized fragments of the cloned inserts by digestion with at least one restriction endonuclease that is different from the first and second restriction endonucleases;

(4) generating a cRNA preparation of antisense cRNA transcripts by incubation of the linearized fragments with a bacteriophage-specific RNA polymerase capable of initiating transcription from the bacteriophage-specific promoter;

(5) dividing the cRNA preparation into sixteen subpools and transcribing first-strand cDNA from each subpool, using a thermostable reverse transcriptase and one of sixteen primers whose 3'-terminus is -N-N, wherein N is one of the four deoxyribonucleotides A, C, G, or T, the primer being at least 15 nucleotides in length, corresponding in sequence to the 3'-end of the bacteriophage-specific promoter, and extending across into at least the first two nucleotides of the cRNA, the mixture including all possibilities for the 3'-terminal two nucleotides;

(6) using the product of transcription in each of the sixteen subpools as a template for a polymerase

of insertion of the cDNA sample in the vector and a

15                   Typically, the anchor primers each have 18 T residues in the tract of T residues, and the stuffer segment of the anchor primers is 14 residues in length. A suitable sequence for the stuffer segment is A-A-C-T-G-G-A-A-G-A-A-T-T-C (SEO ID NO: 1).

Typically, the bacteriophage-specific promoter is selected from the group consisting of T3 promoter and T7 promoter. Most typically, it is the T3 promoter.

Typically, the sixteen primers for priming of transcription of cDNA from crRNA have the sequence A-G-G-

The vector can be the plasmid pBC SK<sup>+</sup> cleaved with ClaI and NotI, in which case the 3'-primer in step (6) can be G-A-A-C-A-A-A-G-C-T-G-G-A-G-C-T-C-C-A-C-C-G-C (SEQ ID NO: 4).

The first restriction endonuclease recognizing a four-nucleotide sequence is typically MspI; alternatively, it can be TagI or HinPII. The restriction endonuclease cleaving at a single site in each of the mixture of anchor primers is typically NotI.

15                   A typical host cell is a strain of Escherichia  
                  coli.

20 (a) dividing the plasmid containing the insert into two fractions, a first fraction cleaved with the restriction endonuclease XhoI and a second fraction cleaved with the restriction endonuclease SalI;

(c) dividing the recombined fractions into thirds and cleaving the first third with the restriction endonuclease HindIII, the second third with the restriction endonuclease BamHI, and the third third with the restriction endonuclease EcoRI; and

(d) recombining the thirds after digestion in order to produce a population of linearized fragments of which about one-sixth of the population corresponds to

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Typically, the step of resolving the polymerase chain reaction amplified fragments by electrophoresis comprises electrophoresis of the fragments on at least two gels.

5

The method can further comprise determining the sequence of the 3'-end of at least one of the mRNAs, such as by:

- (1) eluting at least one cDNA corresponding to  
10 a mRNA from an electropherogram in which bands representing the 3'-ends of mRNAs present in the sample are displayed;
- (2) amplifying the eluted cDNA in a polymerase chain reaction;
- 15 (3) cloning the amplified cDNA into a plasmid;
- (4) producing DNA corresponding to the cloned DNA from the plasmid; and
- (5) sequencing the cloned cDNA.

20

Another aspect of the invention is a method of simultaneous sequence-specific identification of mRNAs corresponding to members of an antisense cRNA pool representing the 3'-ends of a population of mRNAs, the antisense cRNAs that are members of the antisense cRNA  
25 pool being terminated at their 5'-end with a primer sequence corresponding to a bacteriophage-specific vector and at their 3'-end with a sequence corresponding in sequence to a sequence of the vector. The method comprises;

30

- (1) dividing the members of the antisense cRNA pool into sixteen subpools and transcribing first-strand cDNA from each subpool, using a thermostable reverse transcriptase and one of sixteen primers whose 3'-

least 11 nucleotides in length, corresponding in sequence

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to the 3'-end of the bacteriophage-specific promoter, and extending across into at least the first two nucleotides of the cRNA, the mixture including all possibilities for the 3'-terminal two nucleotides;

- 5           (2) using the product of transcription in each of the sixteen subpools as a template for a polymerase chain reaction with a 3'-primer that corresponds in sequence to a sequence vector adjoining the site of insertion of the cDNA sample in the vector and a 5'-  
10 primer selected from the group consisting of: (i) the primer from which first-strand cDNA was made for that subpool; (ii) the primer from which the first-strand cDNA was made for that subpool extended at its 3'-terminus by an additional residue -N, where N can be any of A, C, G,  
15 or T; and (iii) the primer used for the synthesis of first-strand cDNA for that subpool extended at its 3'-terminus by two additional residues -N-N, wherein N can be any of A, C, G, or T, to produce polymerase chain reaction amplified fragments; and  
20           (3) resolving the polymerase chain reaction amplified fragments by electrophoresis to display bands representing the 3'-ends of mRNAs present in the sample.

Yet another aspect of the present invention is  
25 a method for detecting a change in the pattern of mRNA expression in a tissue associated with a physiological or pathological change. This method comprises the steps of:

- (1) obtaining a first sample of a tissue that  
is not subject to the physiological or pathological  
30 change;  
(2) determining the pattern of mRNA expression in the first sample of the tissue by performing steps (1)-(3) of the method described above for simultaneous

ends of a population of mRNAs to generate a first display

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of bands representing the 3'-ends of mRNAs present in the first sample;

(3) obtaining a second sample of the tissue that has been subject to the physiological or  
5 pathological change;

(4) determining the pattern of mRNA expression in the second sample of the tissue by performing steps (1)-(3) of the method described above for simultaneous sequence-specific identification of mRNAs corresponding  
10 to members of an antisense cRNA pool to generate a second display of bands representing the 3'-ends of mRNAs present in the second sample; and

(5) comparing the first and second displays to determine the effect of the physiological or pathological  
15 change on the pattern of mRNA expression in the tissue.

The comparison is typically made in adjacent lanes.

20 The tissue can be derived from the central nervous system or from particular structures within the central nervous system. The tissue can alternatively be derived from another organ or organ system.

25 Another aspect of the present invention is a method of screening for a side effect of a drug. The method can comprise the steps of:

(1) obtaining a first sample of tissue from an organism treated with a compound of known physiological  
30 function;

(2) determining the pattern of mRNA expression in the first sample of the tissue by performing steps (1)-(3) of the method described above for simultaneous



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display of bands representing the 3'-ends of mRNAs present in the first sample;

5 (3) obtaining a second sample of tissue from an organism treated with a drug to be screened for a side effect;

(4) determining the pattern of mRNA expression in the second sample of the tissue by performing steps (1)-(3) of the method described above for simultaneous sequence-specific identification of mRNAs corresponding to members of an antisense cRNA pool to generate a second display of bands representing the 3'-ends of mRNAs present in the second sample; and

15 (5) comparing the first and second displays in order to detect the presence of mRNA species whose expression is not affected by the known compound but is affected by the drug to be screened, thereby indicating a difference in action of the drug to be screened and the known compound and thus a side effect.

20 The drug to be screened can be a drug affecting the central nervous system, such as an antidepressant, a neuroleptic, a tranquilizer, an anticonvulsant, a monoamine oxidase inhibitor, or a stimulant. Alternatively, the drug can be another class of drug such as an anti-parkinsonism agent, a skeletal muscle relaxant, an analgesic, a local anesthetic, a cholinergic, an antispasmodic, a steroid, or a non-steroidal anti-inflammatory drug.

30 Another aspect of the present invention is panels of primers and degenerate mixtures of primers suitable for the practice of the present invention. These include:

NO: 3), wherein N is one of the four deoxyribonucleotides A, C, G, or T;

(2) a panel of primers comprising 64 primers of the sequences A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N-N (SEQ ID NO; 5), wherein N is one of the four deoxyribonucleotides A, C, G, or T;

(3) a panel of primers comprising 256 primers of the sequences A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N-N-N (SEQ ID NO: 6), wherein N is one of the four deoxyribonucleotides A, C, G, or T; and

(4) a panel of primers comprising 12 primers of the sequences A-A-C-T-G-G-A-A-G-A-A-T-T-C-G-C-G-G-C-C-G-C-A-G-G-A-A-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-V-N (SEQ ID NO: 2), wherein V is a deoxyribonucleotide selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from the group consisting of A, C, G, and T; and

(5) a degenerate mixture of primers comprising a mixture of 12 primers of the sequences A-A-C-T-G-G-A-A-G-A-A-T-T-C-G-C-G-G-C-C-G-C-A-G-G-A-A-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-V-N (SEQ ID NO: 2), wherein V is a deoxyribonucleotide selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from the group consisting of A, C, G, and T, each of the 12 primers being present in about an equimolar quantity.

### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, 30 appended claims, and accompanying drawings where:

Figure 1 is a diagrammatic depiction of the method of the present invention showing the various

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Figure 2 is an autoradiogram of a gel showing the result of performing the method of the present invention using several 5'-primers in the PCR step corresponding to known sequences of brain mRNAs and using  
5 liver and brain mRNA as starting material.

#### DESCRIPTION

We have developed a method for simultaneous sequence-specific identification and display of mRNAs in  
10 a mRNA population.

As discussed below, this method has a number of applications in drug screening, the study of physiological and pathological conditions, and genomic  
15 mapping. These applications will be discussed below.

#### I. SIMULTANEOUS SEQUENCE-SPECIFIC IDENTIFICATION OF mRNAs

A method according to the present invention,  
20 based on the polymerase chain reaction (PCR) technique, provides means for visualization of nearly every mRNA expressed by a tissue as a distinct band on a gel whose intensity corresponds roughly to the concentration of the mRNA. The method is based on the observation that  
25 virtually all mRNAs conclude with a 3'-poly (A) tail but does not rely on the specificity of primer binding to the tail.

In general, the method comprises:

30 (1) preparing double-stranded cDNAs from a mRNA population using a mixture of 12 anchor primers, the anchor primers each including: (i) a tract of from 7 to 40 T residues; (ii) a site for cleavage by a restriction

tract of T residues; and a stuffer segment of from 4 to

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- 40 nucleotides, the stuffer segment being located to the 5'-side of the site for cleavage by the restriction endonuclease; and (iv) phasing residues -V-N located at the 3' end of each of the anchor primers, wherein V is a deoxyribonucleotide selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from the group consisting of A, C, G, and T, the mixture including anchor primers containing all possibilities for V and N;
- (2) producing cloned inserts from a suitable host cell that has been transformed by a vector, the vector having the cDNA sample that has been cleaved with a first restriction endonuclease and a second restriction endonuclease inserted therein, the cleaved cDNA sample being inserted in the vector in an orientation that is antisense with respect to a bacteriophage-specific promoter within the vector, the first restriction endonuclease recognizing a four-nucleotide sequence and the second restriction endonuclease cleaving at a single site within each member of the mixture of anchor primers;
- (3) generating linearized fragments of the cloned inserts by digestion with at least one restriction endonuclease that is different from the first and second restriction endonucleases;
- (4) generating a cRNA preparation of antisense cRNA transcripts by incubation of the linearized fragments with a bacteriophage-specific RNA polymerase capable of initiating transcription from the bacteriophage-specific promoter;
- (5) dividing the cRNA preparation into sixteen subpools and transcribing first-strand cDNA from each subpool, using a thermostable reverse transcriptase and one of sixteen primers whose 3'-terminus is -N-N, wherein

corresponding in sequence to the 3'-end of the

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bacteriophage-specific promoter, and extending across into at least the first two nucleotides of the cRNA, the mixture including all possibilities for the 3'-terminal two nucleotides;

- 5           (6) using the product of transcription in each of the sixteen subpools as a template for a polymerase chain reaction with a 3'-primer that corresponds in sequence to a sequence in the vector adjoining the site of insertion of the cDNA sample in the vector and a 5'-  
10 primer selected from the group consisting of: (i) the primer from which first-strand cDNA was made for that subpool; (ii) the primer from which the first-strand cDNA was made for that subpool extended at its 3'-terminus by an additional residue -N, where N can be any of A, C, G,  
15 or T; and (iii) the primer used for the synthesis of first-strand cDNA for that subpool extended at its 3'-terminus by two additional residues -N-N, wherein N can be any of A, C, G, or T, to produce polymerase chain reaction amplified fragments; and  
20           (7) resolving the polymerase chain reaction amplified fragments by electrophoresis to display bands representing the 3'-ends of mRNAs present in the sample.

A depiction of this scheme is shown in Figure  
25 1.

#### A. Isolation of mRNA

The first step in the method is isolation or provision of a mRNA population. Methods of extraction of  
30 RNA are well-known in the art and are described, for example, in J. Sambrook et al., "Molecular Cloning: A Laboratory Manual" (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989), vol. 1, ch. 7.  
reference. Other isolation and extraction methods are

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also well-known. Typically, isolation is performed in the presence of chaotropic agents such as guanidinium chloride or guanidinium thiocyanate, although other detergents and extraction agents can alternatively be used.

Typically, the mRNA is isolated from the total extracted RNA by chromatography over oligo(dT)-cellulose or other chromatographic media that have the capacity to bind the polyadenylated 3'-portion of mRNA molecules. Alternatively, but less preferably, total RNA can be used. However, it is generally preferred to isolate poly(A)<sup>+</sup> RNA.

15           B.   Preparation of Double-Stranded cDNA

Double-stranded cDNAs are then prepared from the mRNA population using a mixture of twelve anchor primers to initiate reverse transcription. The anchor primers each include: (i) a tract of from 7 to 40 T residues; (ii) a site for cleavage by a restriction endonuclease that recognizes more than six bases, the site for cleavage being located to the 5'-side of the tract of T residues; (iii) a stuffer segment of from 4 to 40 nucleotides, the stuffer segment being located to the 5'-side of the site for cleavage by the restriction endonuclease; and (iv) phasing residues -V-N located at the 3' end of each of the anchor primers, wherein V is a deoxyribonucleotide selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from the group consisting of A, C, G, and T. The mixture includes anchor primers containing all possibilities for V and N.

segment of the anchor primers is 14 residues in length.

15 This reaction is carried out under conditions  
for the preparation of double-stranded cDNA from mRNA  
that are well-known in the art. Such techniques are  
described, for example, in Volume 2 of J. Sambrook et  
al., "Molecular Cloning: A Laboratory Manual", entitled  
"Construction and Analysis of cDNA Libraries."  
20 Typically, reverse transcriptase from avian  
myeloblastosis virus is used.

25 The cDNA sample is cleaved with two restriction  
endonucleases. The first restriction endonuclease is an  
endonuclease that recognizes a 4-nucleotide sequence.  
This typically cleaves at multiple sites in most cDNAs.  
The second restriction endonuclease cleaves at a single  
30 site within each member of the mixture of anchor primers.  
Typically, the first restriction endonuclease is MspI and  
the second restriction endonuclease is NotI. The enzyme  
Not does not cleave within most cDNAs. This is desirable

anchor site.

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Alternatively, the first restriction endonuclease can be TaqI or HinP1I. The use of the latter two restriction endonucleases can detect rare mRNAs that are not cleaved by MspI. The first  
5 restriction endonuclease generates a 5'-overhang compatible for cloning into the desired vector, as discussed below. This cloning, for the pBC SK<sup>+</sup> vector, is into the ClaI site, as discussed below.

10 Conditions for digestion of the cDNA are well-known in the art and are described, for example, in J. Sambrook et al., "Molecular Cloning: A Laboratory Manual," Vol. 1, Ch. 5, "Enzymes Used in Molecular Cloning."

15

D. Insertion of Cleaved cDNA into a Vector

The cDNA sample cleaved with the first and second restriction endonucleases is then inserted into a vector. A suitable vector is the plasmid pBC SK<sup>+</sup> that has  
20 been cleaved with the restriction endonucleases ClaI and NotI. The vector contains a bacteriophage-specific promoter. Typically, the promoter is a T3 promoter or a T7 promoter. A preferred promoter is bacteriophage T3 promoter. The cleaved cDNA is inserted into the promoter  
25 in an orientation that is antisense with respect to the bacteriophage-specific promoter.

E. Transformation of a Suitable Host Cell

The vector into which the cleaved DNA has been  
30 inserted is then used to transform a suitable host cell that can be efficiently transformed or transfected by the vector containing the insert. Suitable host cells for cloning are described, for example, in Sambrook et al.,

suitable host cell is a strain of E. coli. A suitable E.



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coli strain is MC1061. Preferably, a small aliquot is also used to transform E. coli strain XL1-Blue so that the percentage of clones with inserts is determined from the relative percentages of blue and white colonies on X-gal plates. Only libraries with in excess of  $5 \times 10^5$  recombinants are typically acceptable.

F. Generation of Linearized Fragments

Plasmid preparations, typically as minipreps, are then made from each of the cDNA libraries. Linearized fragments are then generated by digestion with at least one restriction endonuclease that is different from the first and second restriction endonucleases discussed above. Preferably, an aliquot of each of the cloned inserts is divided into two pools, one of which is cleaved with XhoI and the second with SalI. The pools of linearized plasmids are combined, mixed, then divided into thirds. The thirds are digested with HindIII, BamHI, and EcoRI. This procedure is followed because, in order to generate antisense transcripts of the inserts with T3 RNA polymerase, the template must first be cleaved with a restriction endonuclease that cuts within flanking sequences but not within the inserts themselves. Given that the average length of the 3'-terminal MspI fragments is 256 base pairs, approximately 6% of the inserts contain sites for any enzyme with a hexamer recognition sequence. Those inserts would be lost to further analysis were only a single enzyme utilized. Hence, it is preferable to divide the reaction so that only one of either of two enzymes is used for linearization of each half reaction. Only inserts containing sites for both enzymes (approximately 0.4%) are lost from both halves of the samples. Similarly,

lead to variability in the efficiency of the later

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polymerase chain reactions for different samples because of differential competition for primers. Cleavage of thirds of the samples with one of three enzymes that have single targets in pBC SK<sup>+</sup> between its ClaI and NotI sites eliminates the production of transcripts containing binding sites for the eventual 5' primers in the PCR process from insertless plasmids. The use of three enzymes on thirds of the reaction reduces the use of insert-containing sequences that also contain sites for the enzyme while solving the problem of possible contamination of insertless sequences. If only one enzyme were used, about 10% of the insert-containing sequences would be lost, but this is reduced to about 0.1%, because only those sequences that fail to be cleaved by all three enzymes are lost.

#### G. Generation of cRNA

The next step is a generation of a cRNA preparation of antisense cRNA transcripts. This is performed by incubation of the linearized fragments with an RNA polymerase capable of initiating transcription from the bacteriophage-specific promoter. Typically, as discussed above, the promoter is a T3 promoter, and the polymerase is therefore T3 RNA polymerase. The polymerase is incubated with the linearized fragments and the four ribonucleoside triphosphates under conditions suitable for synthesis.

#### H. Transcription of First-Strand cDNA

The cRNA preparation is then divided into sixteen subpools. First-strand cDNA is then transcribed from each subpool, using a thermostable reverse transcriptase and a primer as described below. A

available from Perkin-Elmer (Norwalk, CT). This enzyme

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is also known as an RNA-dependent DNA polymerase. With this reverse transcriptase, annealing is performed at 60°C, and the transcription reaction at 70°C. This promotes high fidelity complementarity between the primer and the cRNA. The primer used is one of the sixteen primers whose 3'-terminus is -N-N, wherein N is one of the four deoxyribonucleotides A, C, G, or T, the primer being at least 15 nucleotides in length, corresponding in sequence to the 3'-end of the bacteriophage-specific promoter, and extending across into at least the first two nucleotides of the cRNA.

Where the bacteriophage-specific promoter is the T3 promoter, the primers typically have the sequence A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N (SEQ ID NO: 3).

#### I. PCR Reaction

The next step is the use of the product of transcription in each of the sixteen subpools as a template for a polymerase chain reaction with primers as described below to produce polymerase chain reaction amplified fragments.

The primers used are: (a) a 3'-primer that corresponds in sequence to a sequence in the vector adjoining the site of insertion of the cDNA sample in the vector; and (b) a 5'-primer selected from the group consisting of: (i) the primer from which first-strand cDNA was made for that subpool; (ii) the primer from which the first-strand cDNA was made for that subpool extended at its 3'-terminus by an additional residue -N, where N can be any of A, C, G, or T; and (iii) the primer used for the synthesis of first-strand cDNA for that

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When the vector is the plasmid pBC SK<sup>+</sup> cleaved with ClaI and NotI, a suitable 3'-primer is G-A-A-C-A-A-A-A-G-C-T-G-G-A-G-C-T-C-C-A-C-C-G-C (SEQ ID NO: 4).

Where the bacteriophage-specific promoter is the T3 promoter, suitable 5'-primers have the sequences A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N (SEQ ID NO: 3), A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N-N (SEQ ID NO: 5), or A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N-N-N (SEQ ID NO: 6).

Typically, PCR is performed in the presence of <sup>35</sup>S-dATP using a PCR program of 15 seconds at 94°C for denaturation, 15 seconds at 60°C for annealing, and 30 seconds at 72°C for synthesis on a Perkin-Elmer 9600 apparatus (Perkin-Elmer Cetus, Norwalk, CT). The high temperature annealing step minimizes artifactual mispriming by the 5'-primer at its 3'-end and promotes high fidelity copying.

Alternatively, the PCR amplification can be carried out in the presence of a <sup>32</sup>P-labeled deoxyribonucleoside triphosphate, such as [<sup>32</sup>P]dCTP. However, it is generally preferred to use a <sup>35</sup>S-labeled deoxyribonucleoside triphosphate for maximum resolution. Other detection methods, including nonradioactive labels, can also be used.

These series of reactions produces 16, 64, and 256 product pools for the three sets of 5'-primers. It produces 16 product pools for the primer that is the same as the primer from which first-strand cDNA was made. It produces 64 product pools for the primer extended at its 3'-terminus by an additional residue N, where N can be any of the four nucleotides. It produces 256 products

four nucleotides.

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The process of the present invention can be extended by using longer sets of 5'-primers extended at their 3'-end by additional nucleotides. For example, a primer with the 3'-terminus -N-N-N-N-N would give 1024 products.

#### J. Electrophoresis

The polymerase chain reaction amplified fragments are then resolved by electrophoresis to display bands representing the 3'-ends of mRNAs present in the sample.

Electrophoretic techniques for resolving PCR amplified fragments are well-understood in the art and need not be further recited here. The corresponding products are resolved in denaturing DNA sequencing gels and visualized by autoradiography. For the particular vector system described herein, the gels are run so that the first 140 base pairs run off their bottom, since vector-related sequences increase the length of the cDNAs by 140 base pairs. This number can vary if other vector systems are employed, and the appropriate electrophoresis conditions so that vector-related sequences run off the bottom of the gels can be determined from a consideration of the sequences of the vector involved. Typically, each reaction is run on a separate denaturing gel, so that at least two gels are used. It is preferred to perform a series of reactions in parallel, such as from different tissues, and resolve all of the reactions using the same primer on the same gel. A substantial number of reactions can be resolved on the same gel. Typically, as many as thirty reactions can be resolved on the same gel and compared. As discussed below, this provides a way of

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Typically, autoradiography is used to detect the resolved cDNA species. However, other detection methods, such as phosphorimaging or fluorescence, can also be used, and may provide higher sensitivity in certain applications.

According to the scheme, the cDNA libraries produced from each of the mRNA samples contain copies of the extreme 3'-ends from the most distal site for MspI to the beginning of the poly(A) tail of all poly(A)<sup>+</sup> mRNAs in the starting RNA sample approximately according to the initial relative concentrations of the mRNAs. Because both ends of the inserts for each species are exactly defined by sequence, their lengths are uniform for each species allowing their later visualization as discrete bands on a gel, regardless of the tissue source of the mRNA.

The use of successive steps with lengthening primers to survey the cDNAs essentially act like a nested PCR. These steps enhance quality control and diminish the background that potentially could result from amplification of untargeted cDNAs. In a preferred embodiment, the second reverse transcription step subdivides each cRNA sample into sixteen subpools, utilizing a primer that anneals to the sequences derived from pBC SK<sup>+</sup> but extends across the CGG of the non-regenerated MspI site and including two nucleotides (-N-N) of the insert. This step segregates the starting population of potentially 50,000 to 100,000 mRNAs into sixteen subpools of approximately 3,000 to 6,000 members each. In serial iterations of the subsequent PCR step, in which radioactive label is incorporated into the

sixteen subsubpools by using progressively longer

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primers containing three or four nucleotides of the insert.

By first demanding by high temperature  
5 annealing a high fidelity 3'-end match at the reverse  
transcription step in the -N-N positions, and  
subsequently demanding again such high fidelity matching  
into -N-N-N or -N-N-N-N iterations, bleedthrough from  
mismatched priming at the -N-N positions is drastically  
10 minimized.

The steps of the process beginning with  
dividing the cRNA preparation into sixteen subpools and  
transcribing first-strand cDNA from each subpool can be  
15 performed separately as a method of simultaneous  
sequence-specific identification of mRNAs corresponding  
to members of an antisense cRNA pool representing the 3'-  
ends of a population of mRNAs.

20 II. APPLICATIONS OF THE METHOD FOR DISPLAY OF mRNA  
PATTERNS

The method described above for the detection of  
patterns of mRNA expression in a tissue and the resolving  
of these patterns by gel electrophoresis has a number of  
25 applications. One of these applications is its use for  
the detection of a change in the pattern of mRNA  
expression in a tissue associated with a physiological or  
pathological change. In general, this method comprises:

(1) obtaining a first sample of a tissue that  
30 is not subject to the physiological or pathological  
change;

(2) determining the pattern of mRNA expression  
in the first sample of the tissue by performing the

cRNA preparation into sixteen subpools and transcribing first-strand cDNA from each subpool separately as a method of simultaneous sequence-specific identification of mRNAs corresponding to members of an antisense cRNA pool representing the 3'-ends of a population of mRNAs as

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described above to generate a first display of bands representing the 3'-ends of mRNAs present in the first sample;

5 (3) obtaining a second sample of the tissue that has been subject to the physiological or pathological change;

10 (4) determining the pattern of mRNA expression in the second sample of the tissue by performing the method of simultaneous sequence-specific identification of mRNAs corresponding to members of an antisense cRNA pool representing the 3'-ends of a population of mRNAs as described above to generate a second display of bands representing the 3'-ends of mRNAs present in the second sample; and

15 (5) comparing the first and second displays to determine the effect of the physiological or pathological change on the pattern of mRNA expression in the tissue.

20 Typically, the comparison is made in adjacent lanes of a single gel.

The tissue can be derived from the central nervous system. In particular, it can be derived from a structure within the central nervous system that is the retina, cerebral cortex, olfactory bulb, thalamus, 25 hypothalamus, anterior pituitary, posterior pituitary, hippocampus, nucleus accumbens, amygdala, striatum, cerebellum, brain stem, suprachiasmatic nucleus, or spinal cord. When the tissue is derived from the central nervous system, the physiological or pathological change 30 can be any of Alzheimer's disease, parkinsonism, ischemia, alcohol addiction, drug addiction, schizophrenia, amyotrophic lateral sclerosis, multiple

invention can be used to study circadian variation,



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aging, or long-term potentiation, the latter affecting the hippocampus. Additionally, particularly with reference to mRNA species occurring in particular structures within the central nervous system, the method  
5 can be used to study brain regions that are known to be involved in complex behaviors, such as learning and memory, emotion, drug addiction, glutamate neurotoxicity, feeding behavior, olfaction, viral infection, vision, and movement disorders.

10

This method can also be used to study the results of the administration of drugs and/or toxins to an individual by comparing the mRNA pattern of a tissue before and after the administration of the drug or toxin.  
15 Results of electroshock therapy can also be studied.

Alternatively, the tissue can be from an organ or organ system that includes the cardiovascular system, the pulmonary system, the digestive system, the  
20 peripheral nervous system, the liver, the kidney, skeletal muscle, and the reproductive system, or from any other organ or organ system of the body. For example, mRNA patterns can be studied from liver, heart, kidney, or skeletal muscle. Additionally, for any tissue,  
25 samples can be taken at various times so as to discover a circadian effect of mRNA expression. Thus, this method can ascribe particular mRNA species to involvement in particular patterns of function or malfunction.

30

The antisense cRNA pool representing the 3'-ends of mRNAs can be generated by steps (1)-(4) of the method as described above in Section I.

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screening for a side effect of a drug. In general, such a method comprises:

- 5 (1) obtaining a first sample of tissue from an organism treated with a compound of known physiological function;
- (2) determining the pattern of mRNA expression in the first sample of the tissue by performing the method of simultaneous sequence-specific identification of mRNAs corresponding to members of an antisense cRNA  
10 pool representing the 3'-ends of a population of mRNAs, as described above, to generate a first display of bands representing the 3'-ends of mRNAs present in the first sample;
- 15 (3) obtaining a second sample of tissue from an organism treated with a drug to be screened for a side effect;
- (4) determining the pattern of mRNA expression in the second sample of the tissue by performing the method of simultaneous sequence-specific identification  
20 of mRNAs corresponding to members of an antisense cRNA pool representing the 3'-ends of a population of mRNAs, as described above, to generate a second display of bands representing the 3'-ends of mRNAs present in the second sample; and
- 25 (5) comparing the first and second displays in order to detect the presence of mRNA species whose expression is not affected by the known compound but is affected by the drug to be screened, thereby indicating a difference in action of the drug to be screened and the  
30 known compound and thus a side effect.

In particular, this method can be used for drugs affecting the central nervous system, such as

stimulants. However, this method can in fact be used for

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any drug that may affect mRNA expression in a particular tissue. For example, the effect on mRNA expression of anti-parkinsonism agents, skeletal muscle relaxants, analgesics, local anesthetics, cholinergics,  
5 antispasmodics, steroids, non-steroidal anti-inflammatory drugs, antiviral agents, or any other drug capable of affecting mRNA expression can be studied, and the effect determined in a particular tissue or structure.

10 A further application of the method of the present invention is in obtaining the sequence of the 3'-ends of mRNA species that are displayed. In general, a method of obtaining the sequence comprises:

- 15 (1) eluting at least one cDNA corresponding to a mRNA from an electropherogram in which bands representing the 3'-ends of mRNAs present in the sample are displayed;
- (2) amplifying the eluted cDNA in a polymerase chain reaction;
- 20 (3) cloning the amplified cDNA into a plasmid;
- (4) producing DNA corresponding to the cloned DNA from the plasmid; and
- (5) sequencing the cloned cDNA.

25 The cDNA that has been excised can be amplified with the primers previously used in the PCR step. The cDNA can then be cloned into pCR II (Invitrogen, San Diego, CA) by TA cloning and ligation into the vector. Minipreps of the DNA can then be produced by standard  
30 techniques from subclones and a portion denatured and split into two aliquots for automated sequencing by the dideoxy chain termination method of Sanger. A commercially available sequencer can be used, such as a

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cDNAs studied, in the length range of 50-500 bp, across the entire length of the fragment.

5        These partial sequences can then be used to  
scan genomic data bases such as GenBank to recognize  
sequence identities and similarities using programs such  
as BLASTN and BLASTX. Because this method generates  
sequences from only the 3'-ends of mRNAs it is expected  
that open reading frames (ORFs) would be encountered only  
10 occasionally, as the 3'-untranslated regions of brain  
mRNAs are on average longer than 1300 nucleotides (J.G.  
Sutcliffe, supra). Potential ORFs can be examined for  
signature protein motifs.

15        The cDNA sequences obtained can then be used to  
design primer pairs for semiquantitative PCR to confirm  
tissue expression patterns. Selected products can also  
be used to isolate full-length cDNA clones for further  
analysis. Primer pairs can be used for SSCP-PCR (single  
20 strand conformation polymorphism-PCR) amplification of  
genomic DNA. For example, such amplification can be  
carried out from a panel of interspecific backcross mice  
to determine linkage of each PCR product to markers  
already linked. This can result in the mapping of new  
25 genes and can serve as a resource for identifying  
candidates for mapped mouse mutant loci and homologous  
human disease genes. SSCP-PCR uses synthetic  
oligonucleotide primers that amplify, via PCR, a small  
(100-200 bp) segment. (M. Orita et al., "Detection of  
30 Polymorphisms of Human DNA by Gel Electrophoresis as  
Single-Strand Conformation Polymorphisms," Proc. Natl.  
Acad. Sci. USA 86: 2766-2770 (1989); M. Orita et al.,  
"Rapid and Sensitive Detection of Point Mutations in DNA

10

### III. PANELS AND DEGENERATE MIXTURES OF PRIMERS

[illegible]

G-A-A-T-T-C-G-C-G-G-C-C-G-C-A-G-G-A-A-T-T-T-T-T-T-T-T-  
T-T-T-T-T-T-T-T-T-V-N (SEQ ID NO: 2), wherein V is a  
deoxyribonucleotide selected from the group consisting of  
A, C, and G; and N is a deoxyribonucleotide selected from  
the group consisting of A, C, G, and T, each of the 12  
primers being present in about an equimolar quantity.

### EXAMPLE

To demonstrate the effectiveness of the method of the present invention, it was applied using 5'-primers extended at their 3'-ends by two nucleotides and corresponding to the sequence of known brain mRNAs of different concentrations, such as neuron-specific enolase (NSE) at roughly 0.5% concentration (S. Forss-Petter et al., "Neuron-Specific Enolase: Complete Structure of Rat mRNA, Multiple Transcriptional Start Sites and Evidence for Translational Control," J. Neurosci. Res. 16: 141-156 (1986)), RC3 at about 0.01%, and somatostatin at 0.001% (G.H. Travis & J.G. Sutcliffe, "Phenol Emulsion-Enhanced DNA-Driven Subtractive cDNA Cloning: Isolation of Low-Abundance Monkey Cortex-Specific mRNAs," Proc. Natl. Acad. Sci. USA 85: 1696-1700 (1988)) to compare cDNAs made from libraries constructed from cerebral cortex, striatum, cerebellum and liver RNAs made as described

were absolutely reproducible in duplicate samples.

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Approximately two-thirds of the bands differed between brain and liver samples, including the bands of the correct lengths corresponding to the known brain-specific mRNAs. This was confirmed by excision of the bands from the gels, amplification and sequencing. Only a few bands differed among samples for various brain regions for any particular primer, although some band intensities differed.

10           The band corresponding to NSE, a relatively prevalent mRNA species, appeared in all of the brain samples but not in the liver samples, but was not observed when any of the last three single nucleotides within the four-base 3'-terminal sequence -N-N-N-N was  
15           changed in the synthetic 5'-primer. When the first N was changed, a small amount of bleedthrough is detected. For the known species, the intensity of the autoradiographic signal was roughly proportional to mRNA prevalence, and mRNAs with concentrations of one part in  $10^5$  or greater of  
20           the poly(A)<sup>+</sup> RNA were routinely visible, with the occasional problem that cDNAs that migrated close to more intense bands were obscured.

          A sample of the data is shown in Figure 2. In  
25           the 5 gel lanes on the left, cortex cRNA was substrate for reverse transcription with the primer A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N (SEQ ID NO: 3) where -N-N is -C-T (primer 118), -G-T (primer 116) or -C-G (primer 106). The PCR amplification used primers A-G-G-T-C-G-A-C-G-G-T-  
30           A-T-C-G-G-N-N-N-N (SEQ ID NO: 6) where -N-N-N-N is -C-T-A-C (primer 128), -C-T-G-A (primer 127), -C-T-G-C (primer 111), -G-T-G-C (primer 134), and -C-G-G-C (primer 130), as indicated in Figure 2. Primers 118 and 111 match the  
          downstream from the NSE site located the nearest 3' end of the NSE mRNA sequence. Primer 127 is mismatched

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with the NSE sequence in the last (-1) position, primer 128 in the next-to-last (-2) position, primers 106 and 130 in the -3 position, and primers 116 and 134 in the -4 position. Primer 134 extended two nucleotides further upstream than the others shown here, hence its PCR products are two nucleotides longer relative to the products in other lanes.

In each lane, 50-100 bands were visible in 15-minute exposures using  $^{32}\text{P}$ -dCTP to radiolabel the products. These bands were apparently distinct for each primer pair, with the exception that a subset of the 118-111 bands appeared more faintly in the 116-134 lane, trailing by two nucleotides, indicating bleedthrough in the four position.

The 118-111 primer set was used again on separate cortex (CX) and liver (LV) cRNAs. The cortex pattern was identical to that in lane 118-111, demonstrating reproducibility. The liver pattern differed from CX in the majority of species. The asterisk indicates the position of the NSE product. Analogous primer sets detected RC3 and somatostatin (somat) products (asterisks) in CX but not LV lanes. The relative band intensities of a given PCR product can be compared within lanes using the same primer set, but not different sets.

This example demonstrates the feasibility and reproducibility of the method of the present invention and its ability to resolve different mRNAs. It further demonstrates that prevalence of particular mRNA species can be estimated from the intensity of the

in both high and low concentrations simultaneously.



ADVANTAGES OF THE PRESENT INVENTION

The present method can be used to identify genes whose expression is altered during neuronal development, in models of plasticity and regeneration, in response to chemical or electrophysiological challenges such as neurotoxicity and long-term potentiation, and in response to behavioral, viral, drug/alcohol paradigms, the occurrence of cell death or apoptosis, aging, pathological conditions, and other conditions affecting mRNA expression. Although the method is particularly useful for studying gene expression in the nervous system, it is not limited to the nervous system and can be used to study mRNA expression in any tissue. The method allows the visualization of nearly every mRNA expressed by a tissue as a distinct band on a gel whose intensity corresponds roughly to the concentration of the mRNA.

The method has the advantage that it does not depend on potentially irreproducible mismatched random priming, so that it provides a high degree of accuracy and reproducibility. Moreover, it reduces the complications and imprecision generated by the presence of concurrent bands of different length resulting from the same mRNA species as the result of different priming events. In methods using random priming, such concurrent bands can occur and are more likely to occur for mRNA species of high prevalence. In the present method, such concurrent bands are avoided.

The method provides sequence-specific information about the mRNA species and can be used to generate primers, probes, and other specific sequences.

described in considerable detail, with reference to certain preferred versions thereof, other versions are

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possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of the preferred versions contained herein.

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## SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Method for Simultaneous  
Identification of Differentially Expressed mRNAs and  
Measurement of Relative Concentrations

(iii) NUMBER OF SEQUENCES: 6

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT US94/  
(B) FILING DATE: 14-NOV-1994  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/152,482  
(B) FILING DATE: 12-NOV-1993  
(C) CLASSIFICATION: 435

## 1. SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

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(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AACTGGAAGA ATTC

14

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AACTGGAAGA ATTCGCGGCC GCAGGAATTT TTTTTTTTTT TTTTTVN

47

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

-43-

(A) ORGANISM: Synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGGTCGACGG TATCGGNN

18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAACAAAAGC TGGAGCTCCA CCGC

24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGTCGACGG TATCGGNNN

19

(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid

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(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
    (A) ORGANISM: Synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGGTCGACGG TATCGGNNNN

20

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We claim:

1. A method for simultaneous sequence-specific identification of mRNAs in a mRNA population comprising the steps of:

(a) preparing double-stranded cDNAs from a mRNA population using a mixture of 12 anchor primers, the anchor primers each including: (i) a tract of from 7 to 40 T residues; (ii) a site for cleavage by a restriction endonuclease that recognizes more than six bases, the site for cleavage being located to the 5'-side of the tract of T residues; (iii) a stuffer segment of from 4 to 40 nucleotides, the stuffer segment being located to the 5'-side of the site for cleavage by the restriction endonuclease; and (iv) phasing residues -V-N located at the 3' end of each of the anchor primers, wherein V is a deoxyribonucleotide selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from the group consisting of A, C, G, and T, the mixture including anchor primers containing all possibilities for V and N;

(b) producing cloned inserts from a suitable host cell that has been transformed by a vector, the vector having the cDNA sample that has been cleaved with a first restriction endonuclease and a second restriction endonuclease inserted therein, the cleaved cDNA sample being inserted in the vector in an orientation that is antisense with respect to a bacteriophage-specific promoter within the vector, the first restriction endonuclease recognizing a four-nucleotide sequence and the second restriction endonuclease cleaving at a single site within each member of the mixture of anchor primers;

(c) generating linearized fragments of the

restriction endonucleases;

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- (d) generating a cRNA preparation of antisense cRNA transcripts by incubation of the linearized fragments with a bacteriophage-specific RNA polymerase capable of initiating transcription from the
- 5 bacteriophage-specific promoter;
- (e) dividing the cRNA preparation into sixteen subpools and transcribing first-strand cDNA from each subpool, using a thermostable reverse transcriptase and one of sixteen primers whose 3'-terminus is -N-N, wherein
- 10 N is one of the four deoxyribonucleotides A, C, G, or T, the primer being at least 15 nucleotides in length, corresponding in sequence to the 3'-end of the bacteriophage-specific promoter, and extending across into at least the first two nucleotides of the cRNA, the
- 15 mixture including all possibilities for the 3'-terminal two nucleotides;
- (f) using the product of transcription in each of the sixteen subpools as a template for a polymerase chain reaction with a 3'-primer that corresponds in
- 20 sequence to a sequence in the vector adjoining the site of insertion of the cDNA sample in the vector and a 5'-primer selected from the group consisting of: (i) the primer from which first-strand cDNA was made for that subpool; (ii) the primer from which the first-strand cDNA
- 25 was made for that subpool extended at its 3'-terminus by an additional residue -N, where N can be any of A, C, G, or T; and (iii) the primer used for the synthesis of first-strand cDNA for that subpool extended at its 3'-terminus by two additional residues -N-N, wherein N can
- 30 be any of A, C, G, or T, to produce polymerase chain reaction amplified fragments; and
- (g) resolving the polymerase chain reaction amplified fragments by electrophoresis to display bands



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2. The method of claim 1 wherein the anchor primers each have 18 T residues in the tract of T residues.

5 3. The method of claim 1 wherein the stuffer segment of the anchor primers is 14 residues in length.

4. The method of claim 3 wherein the sequence of the stuffer segment is A-A-C-T-G-G-A-A-G-A-A-T-T-C  
10 (SEQ ID NO: 1).

5. The method of claim 1 wherein the site for cleavage by a restriction endonuclease that recognizes more than six bases is the NotI cleavage site.  
15

6. The method of claim 4 wherein the anchor primers have the sequence A-A-C-T-G-G-A-A-G-A-A-T-T-C-G-C-G-G-C-C-G-C-A-G-G-A-A-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-V-N (SEQ ID NO: 2).  
20

7. The method of claim 1 wherein the bacteriophage-specific promoter is selected from the group consisting of T3 promoter and T7 promoter.

25 8. The method of claim 7 wherein the bacteriophage-specific promoter is T3 promoter.

9. The method of claim 8 wherein the sixteen primers for priming of transcription of cDNA from cRNA  
30 have the sequence A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N (SEQ ID NO: 3).

10. The method of claim 1 wherein the vector  
T-C-C-A-C-C-G-C (SEQ ID NO: 4).

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11. The method of claim 1 wherein the first restriction endonuclease recognizing a four-nucleotide sequence is MspI.

5           12. The method of claim 1 wherein the first restriction endonuclease recognizing a four-nucleotide sequence is selected from the group consisting of TaqI and HinPII.

10           13. The method of claim 1 wherein the restriction endonuclease cleaving at a single site in each of the mixture of anchor primers is NotI.

15           14. The method of claim 1 wherein the step of generating linearized fragments of the cloned inserts comprises:

(i) dividing the plasmid containing the insert into two fractions, a first fraction cleaved with the restriction endonuclease XhoI and a second fraction  
20           cleaved with the restriction endonuclease SalI;

(ii) recombining the first and second fractions after cleavage;

(iii) dividing the recombined fractions into thirds and cleaving the first third with the  
25           restriction endonuclease HindIII, the second third with the restriction endonuclease BamHI, and the third third with the restriction endonuclease EcoRI; and

(iv) recombining the thirds after digestion in order to produce a population of linearized  
30           fragments of which about one-sixth of the population corresponds to the product of cleavage by each of the possible combinations of enzymes.

species.

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16. The method of claim 1 wherein the intensity of each band displayed after electrophoresis is about proportional to the abundance of the mRNA corresponding to the band in the original mixture.

5

17. The method of claim 16 further comprising a step of determining the relative abundance of each mRNA in the original mixture from the intensity of the band corresponding to that mRNA after electrophoresis.

10

18. The method of claim 1 wherein the step of resolving the polymerase chain reaction amplified fragments by electrophoresis comprises electrophoresis of the fragments on at least two gels.

15

19. The method of claim 1 wherein the suitable host cell is Escherichia coli.

20. The method of claim 1 further comprising the steps of:

(h) eluting at least one cDNA corresponding to a mRNA from an electropherogram in which bands representing the 3'-ends of mRNAs present in the sample are displayed;

25 (i) amplifying the eluted cDNA in a polymerase chain reaction;

(j) cloning the amplified cDNA into a plasmid;

(k) producing DNA corresponding to the cloned DNA from the plasmid; and

30 (l) sequencing the cloned cDNA.

21. A method for simultaneous sequence-specific identification of mRNAs in a mRNA population

comprising the steps of:

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(b) preparing double-stranded cDNAs from the mRNA population using a mixture of 12 anchor primers with the sequence A-A-C-T-G-G-A-A-G-A-A-T-T-C-G-C-G-G-C-C-G-C-A-G-G-A-A-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-V-N (SEQ ID NO: 2), wherein V is a deoxyribonucleotide selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from the group consisting of A, C, G, and T, the mixture including anchor primers containing all possibilities for V and N, to produce a cDNA sample;

(c) cleaving the cDNA sample with two restriction endonucleases, a first restriction endonuclease MspI and a second restriction endonuclease NotI;

(d) inserting the cDNA sample cleaved with the first and second restriction endonucleases into a vector, the cleaved cDNA being inserted in an orientation that is antisense with respect to a T3 promoter within the vector, the vector being the plasmid pBC SK<sup>+</sup> cleaved with ClaI and NotI;

(e) transforming Escherichia coli with the vector into which the cleaved cDNA has been inserted to produce cloned inserts;

(f) generating linearized fragments of the cloned inserts by digestion with at least one restriction endonuclease that is different from the first and second restriction endonucleases;

(g) generating a cRNA preparation of antisense cRNA transcripts by incubation of the linearized fragments with a T3 RNA polymerase capable of initiating transcription from the T3-specific promoter;

(h) dividing the cRNA preparation into sixteen subpools and transcribing first-strand cDNA from each

G-N-N (SEQ ID NO: 3), wherein N is one of the four

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deoxyribonucleotides A, C, G, or T, the mixture including all possibilities for the 3'-terminal two nucleotides;

(i) using the product of transcription in each of the sixteen subpools as a template for a polymerase chain reaction with the 3'-primer G-A-A-C-A-A-A-A-G-C-T-G-G-A-G-C-T-C-C-A-C-C-G-C (SEQ ID NO: 4), and a 5'-primer selected from the group consisting of: (1) the primer from which first-strand cDNA was made for that subpool; (2) the primer from which the first-strand cDNA was made for that subpool extended at its 3'-terminus by an additional residue -N, where N can be any of A, C, G, or T; and (3) the primer used for the synthesis of first-strand cDNA for that subpool extended at its 3'-terminus by two additional residues -N-N, wherein N can be any of A, C, G, or T, to produce polymerase chain reaction amplified fragments; and

(j) resolving the polymerase chain reaction amplified fragments by electrophoresis to display bands representing the 3'-ends of mRNAs present in the sample.

20

22. A method of simultaneous sequence-specific identification of mRNAs corresponding to members of an antisense cRNA pool representing the 3'-ends of a population of mRNAs, the antisense cRNAs that are members of the antisense cRNA pool being terminated at their 5'-end with a primer sequence corresponding to a bacteriophage-specific vector and at their 3'-end with a sequence corresponding in sequence to a sequence of the vector, the method comprising;

25

(a) dividing the members of the antisense cRNA pool into sixteen subpools and transcribing first-strand cDNA from each subpool, using a thermostable reverse transcriptase and one of sixteen primers whose 3'

deoxyribonucleotides A, C, G, or T, the primer being at least 15 nucleotides in length, corresponding in sequence

30

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to the 3'-end of the bacteriophage-specific promoter, and extending across into at least the first two nucleotides of the cRNA, the mixture including all possibilities for the 3'-terminal two nucleotides;

- 5 (b) using the product of transcription in each of the sixteen subpools as a template for a polymerase chain reaction with a 3'-primer that corresponds in sequence to a sequence vector adjoining the site of insertion of the cDNA sample in the vector and a 5'-  
10 primer selected from the group consisting of: (i) the primer from which first-strand cDNA was made for that subpool; (ii) the primer from which the first-strand cDNA was made for that subpool extended at its 3'-terminus by an additional residue -N, where N can be any of A, C, G,  
15 or T; and (iii) the primer used for the synthesis of first-strand cDNA for that subpool extended at its 3'-terminus by two additional residues -N-N, wherein N can be any of A, C, G, or T, to produce polymerase chain reaction amplified fragments; and  
20 (c) resolving the polymerase chain reaction amplified fragments by electrophoresis to display bands representing the 3'-ends of mRNAs present in the sample.

23. A method for detecting a change in the  
25 pattern of mRNA expression in a tissue associated with a physiological or pathological change comprising the steps of:

- (a) obtaining a first sample of a tissue that  
is not subject to the physiological or pathological  
30 change;  
(b) determining the pattern of mRNA expression in the first sample of the tissue by performing steps (a)-(c) of claim 22 to generate a first display of bands

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(c) obtaining a second sample of the tissue that has been subject to the physiological or pathological change;

5 (d) determining the pattern of mRNA expression in the second sample of the tissue by performing steps (a)-(c) of claim 22 to generate a second display of bands representing the 3'-ends of mRNAs present in the second sample; and

10 (e) comparing the first and second displays to determine the effect of the physiological or pathological change on the pattern of mRNA expression in the tissue.

15 24. The method of claim 23 wherein the tissue is derived from the central nervous system.

25 25. The method of claim 24 wherein the physiological or pathological change is selected from the group consisting of Alzheimer's disease, parkinsonism, ischemia, alcohol addiction, drug addiction, schizophrenia, amyotrophic lateral sclerosis, multiple sclerosis, depression, and bipolar manic-depressive disorder.

25 26. The method of claim 24 wherein the physiological or pathological change is associated with learning or memory, emotion, glutamate neurotoxicity, feeding behavior, olfaction, vision, movement disorders, viral infection, electroshock therapy, or the administration of a drug or toxin.

30

27. The method of claim 24 wherein the physiological or pathological change is selected from the group consisting of circadian variation, aging, and long

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28. The method of claim 24 wherein the tissue is derived from a structure within the central nervous system selected from the group consisting of retina, cerebral cortex, olfactory bulb, thalamus, hypothalamus, anterior pituitary, posterior pituitary, hippocampus, nucleus accumbens, amygdala, striatum, cerebellum, brain stem, suprachiasmatic nucleus, and spinal cord.

29. The method of claim 23 wherein the tissue is from an organ or organ system selected from the group consisting of the cardiovascular system, the pulmonary system, the digestive system, the peripheral nervous system, the liver, the kidney, skeletal muscle, and the reproductive system.

30. A method of screening for a side effect of a drug comprising the steps of:

(a) obtaining a first sample of tissue from an organism treated with a compound of known physiological function;

(b) determining the pattern of mRNA expression in the first sample of the tissue by performing steps (a)-(c) of claim 22 to generate a first display of bands representing the 3'-ends of mRNAs present in the first sample;

(c) obtaining a second sample of tissue from an organism treated with a drug to be screened for a side effect;

(d) determining the pattern of mRNA expression in the second sample of the tissue by performing steps (a)-(c) of claim 22 to generate a second display of bands representing the 3'-ends of mRNAs present in the second sample; and

expression is not affected by the known compound but is



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affected by the drug to be screened, thereby indicating a difference in action of the drug to be screened and the known compound and thus a side effect.

5           31. The method of claim 30 wherein the drug to be tested is selected from the group consisting of antidepressants, neuroleptics, tranquilizers, anticonvulsants, monoamine oxidase inhibitors, and stimulants.

10           32. The method of claim 30 wherein the drug to be tested is selected from the group consisting of anti-parkinsonism agents, skeletal muscle relaxants, analgesics, local anesthetics, cholinergics, antiviral  
15 agents, antispasmodics, steroids, and non-steroidal anti-inflammatory drugs.

          33. A panel of primers comprising 16 primers of the sequence A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N (SEQ  
20 ID NO: 3), wherein N is one of the four deoxyribonucleotides A, C, G, or T.

          34. A panel of primers comprising 64 primers of the sequences A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N-N  
25 (SEQ ID NO: 5), wherein N is one of the four deoxyribonucleotides A, C, G, or T.

          35. A panel of primers comprising 256 primers of the sequences A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-N-N-N-N  
30 (SEQ ID NO: 6), wherein N is one of the four deoxyribonucleotides A, C, G, or T.

          36. A panel of primers comprising 16 primers of the sequence A-G-G-T-C-G-A-C-G-G-T-A-T-C-G-G-V (SEQ ID NO: 2), wherein V is a deoxyribonucleotide

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selected from the group consisting of A, C, and G; and N is a deoxyribonucleotide selected from the group consisting of A, C, G, and T.

- 5                    37. A degenerate mixture of primers comprising  
a mixture of 12 primers of the sequences A-A-C-T-G-G-A-A-  
G-A-A-T-T-C-G-C-G-G-C-C-G-C-A-G-G-A-A-T-T-T-T-T-T-T-T-  
T-T-T-T-T-T-T-T-T-V-N (SEQ ID NO: 2), wherein V is a  
deoxyribonucleotide selected from the group consisting of  
10 A, C, and G; and N is a deoxyribonucleotide selected from  
the group consisting of A, C, G, and T, each of the 12  
primers being present in about an equimolar quantity.

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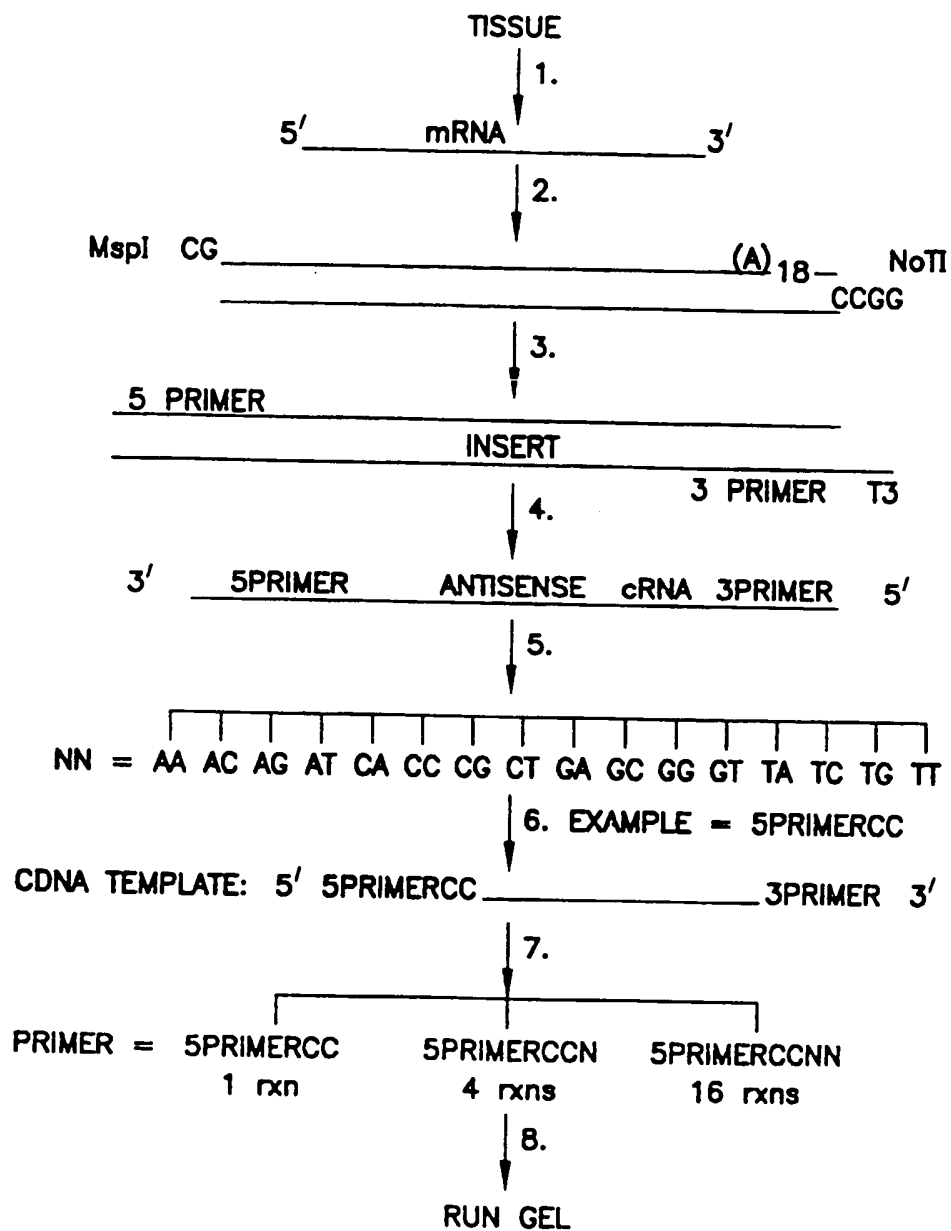


FIG 1

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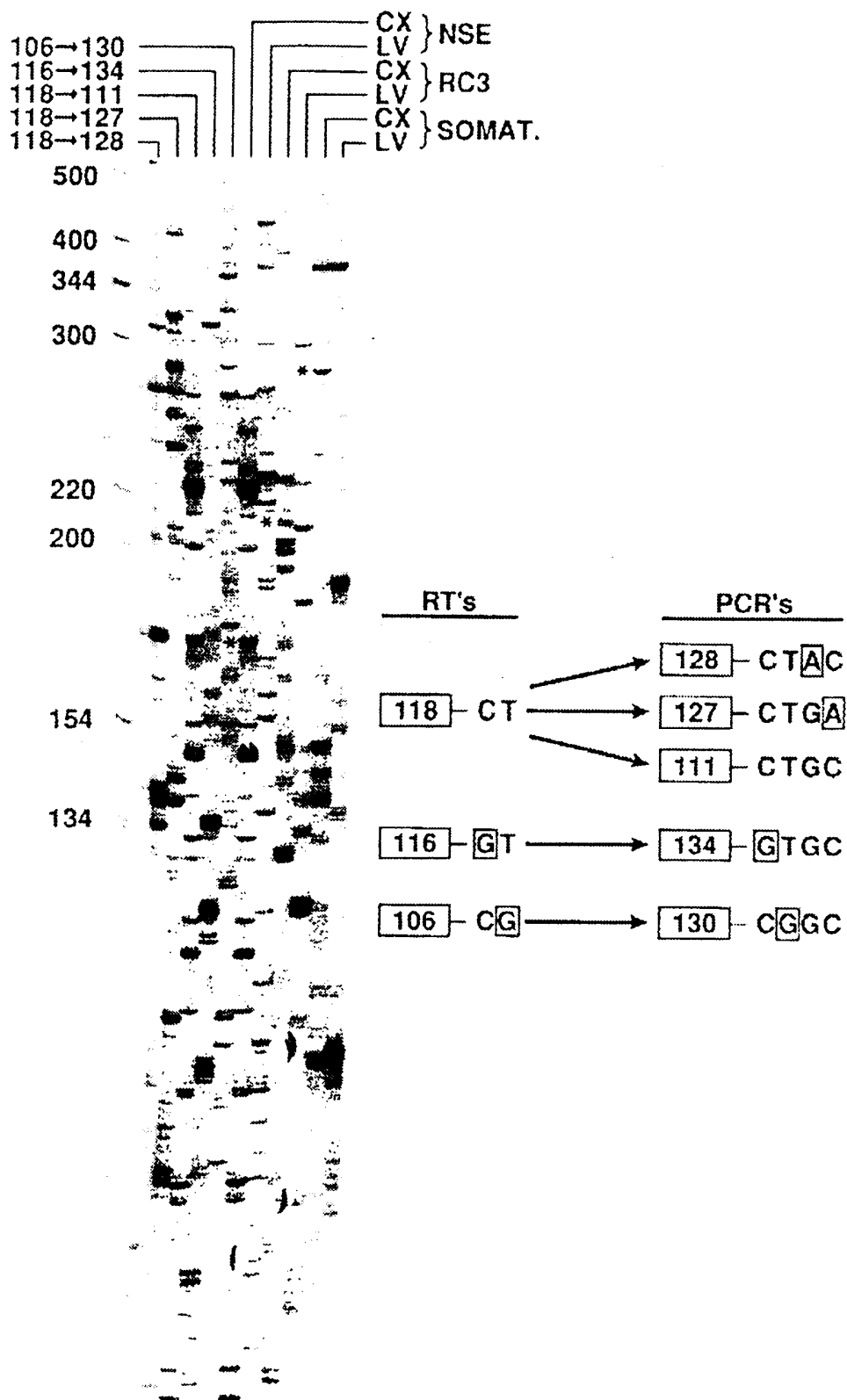


FIG. 2

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/13041

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/11; C12P 19/34; C12Q 1/68

US CL : 435/6, 91.1, 91.3; 536/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.3; 536/24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

search terms: PCR, mRNA, display, pattern

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STRATAGENE CLONING SYSTEMS, Product Catalog, published 1993, page 312, see entire product description.	33-35
Y	Science, Volume 257, issued 14 August 1992, Liang et al, "Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction", pages 967-971, see entire article.	36, 37
Y	Nucleic Acids Research, Volume 21, Number 4, issued 1993, Liang et al, "Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization", pages 3269-3275, see entire article.	36, 37

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

Date of mailing of the international search report

SEARCH REPORT

11 FEB 1995

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/13041

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Trends in Genetics, Volume 5, Number 6, issued June 1989, White et al, "The polymerase chain reaction", pages 185-189, see entire article.	36, 37